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Durable protection of rhesus macaques immunized with a replicating adenovirus-SIV multigene prime/protein boost vaccine regimen against a second SIV_{mac251} rectal challenge: Role of SIV-specific CD8+ T cell responses

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Abstract

Previously, priming with replication-competent adenovirus-SIV multigenic vaccines and boosting with envelope subunits strongly protected 39% of rhesus macaques against rectal SIV_{mac251} challenge. To evaluate protection durability, eleven of the protected and two SIV-infected unimmunized macaques that controlled viremia were re-challenged rectally with SIV_{mac251}. Strong protection was observed in 8/11 vaccinees, including two exhibiting <50 SIV RNA copies. Decreased viremia compared to naïve controls was observed in the other three. The SIV-infected unimmunized macaques modestly controlled viremia but exhibited CD4 counts ≤200, unlike the protected macaques. Durable protection was associated with significantly increased SIV-specific ELISPOT responses and lymphoproliferative responses to p27 at re-challenge. After CD8 depletion, 2 of 8 re-challenged, protected vaccinees maintained <50 SIV RNA copies; SIV RNA emerged in 6. Re-appearance of CD8 cells and restoration of SIV-specific cellular immunity coincided with viremia suppression. Overall, cellular immunity induced by vaccination and/or low-level, inapparent viremia post-first SIV_{mac251} challenge, was associated with durable protection against re-challenge.
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Keywords: AIDS; HIV; SIV; Adenovirus; Rhesus macaque; Cellular immunity

Introduction

An efficient AIDS vaccine will require long-lasting immunity in order to protect against repeated HIV exposures. Live, attenuated SIV vaccines provide low-level expression of viral proteins, thus inducing vigorous antiviral immunity that results in effective protection against repeated challenges with pathogenic SIV (Connor et al., 1998; Daniel et al., 1992; Stebbings et al., 2002). However, some studies have shown that infection with attenuated SIV leads to a high level of viremia and disease progression in infant and adult macaques (Baba et al., 1995, 1999; Gundluch et al., 2000; Hofmann-Lehmann et al., 2003). Unless such attenuated viruses can be appropriately engineered and proven to be safe, the next best option may be provided by live, replicating vectors (Malkevitch and Robert-Guroff, 2004). We have been developing replication-competent adenovirus (Ad) vectors as vehicles for AIDS vaccines. Our studies in both chimpanzee and rhesus macaque models have demonstrated that priming with replicating Ad recombinants encoding HIV or SIV genes

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followed by boosting with viral protein subunits elicits potent humoral, cellular, and mucosal immune responses (Buge et al., 1997, 1999; Lubeck et al., 1997; Malkevitch et al., 2003; Patterson et al., 2003; Peng et al., 2005; Pinczewski et al., 2005; Zhao et al., 2003a; Zolla-Pazner et al., 1998). Protective efficacy has been demonstrated in chimpanzees against low- and high-dose HIV challenges, including challenge with a primary isolate heterologous to the vaccine strain (Lubeck et al., 1997; Robert-Guroff et al., 1998). In macaques, decreased acute viremia following intravaginal challenge with virulent SIV_{mac251} resulted from a vaccine regimen using only an Ad-SIV*env* recombinant and gp120 boosting (Buge et al., 1997). A greater degree of protection against an intrarectal SIV_{mac251} challenge was achieved with the addition of an Ad-SIV*gag* recombinant to the immunization strategy (Zhao et al., 2003b). Most recently, we reported that two sequential immunizations with Ad type 5 host-range mutant (Ad5hr)-SIV multigene recombinants encoding SIV*env/rev*, *gag*, and/or *nef*, followed by two envelope subunit boosts elicited potent, persistent cellular and humoral immunity (Malkevitch et al., 2003; Patterson et al., 2003) that lasted more than 30 weeks. Following SIV_{mac251} challenge, decreased acute viremia was associated with anti-envelope binding antibodies, and decreased chronic phase viremia with SIV-specific cellular immune responses to Env and Rev (Patterson et al., 2004). Although we were not able to demonstrate neutralization of primary SIV_{mac251} by sera obtained from the vaccinated macaques at the time of challenge, antibodies present in the sera mediated potent antibody-dependent cellular cytotoxicity (ADCC). Antibody titers mediating ADCC were correlated with reduced acute viral burdens (Gómez-Román et al., 2005). The striking result of this study was the subset of macaques (39%) that exhibited remarkably strong protection, either showing no viremia, clearing viremia, or controlling viremia at the threshold of detection (Patterson et al., 2004).

One rationale for choice of a replicating vaccine vector is its potential for eliciting long-lasting protection. Therefore, in the present work, we addressed this point and evaluated the durability of protection in the subset of immunized, highly protected macaques by administering a second pathogenic SIV_{mac251} rectal challenge 1 year after the first challenge with no intervening immunizations. Two SIV-infected, unimmunized macaques and 2 naïve control macaques were included in the study. We systematically monitored immune responses in the monkeys before and after the rectal challenge. Strong, durable protection was seen in 8 of 11 vaccinees associated with a high level of SIV-specific IFN-γ-secreting cells primarily to Gag and elevated lymphoproliferative responses to p27 prior to re-challenge compared to the naïve and unimmunized viremic animals. Subsequent in vivo depletion of CD8+ T cells of the 8 durably protected macaques together with careful monitoring of SIV-specific immune responses suggested a protective role of SIV-specific CD8+ T cells, although a contribution of NK or NKT cells to this protection cannot be excluded. SIV-specific antibodies, although present, were not correlated with protection from the second SIV_{mac251} intrarectal challenge. Our study demonstrates that the vaccine regimen performed as desired by eliciting potent, SIV-specific CD8+ T lymphocytes that initially contained chronic viral burdens following the first SIV_{mac251} challenge (Patterson et al., 2004). This cellular immunity, perhaps augmented by immune responses resulting from subsequent low-level or inapparent SIV infection, led to long-term effective control and/or clearance of viremia following the second viral challenge.

Results

Durable protection in immunized macaques after a second SIV_{mac251} challenge

As previously reported (Patterson et al., 2004), a vaccine regimen composed of priming with two or more Ad5hr-SIV recombinants and boosting with SIVgp120 or priming with Ad5hr-SIV*env/rev* and boosting with an SIV polypeptide (termed "peptomer") representing the CD4 binding region of the SIV envelope, resulted in strong protection in 12 of 31

Table 1
Immunization, challenge history, and second challenge of macaques in the study

Macaque	Ad5hr-SIV recombinant prime[a] (weeks 0, 12) encoding:	Envelope subunit boost[b] (weeks 24 and 36)	1st IR challenge (week 42)	2nd IR challenge (week 105[c])
Macaques previously protected against 1st challenge				
5	SIV*env/rev*	SIV peptomer	SIV_{mac251}	SIV_{mac251}
7	”	”	”	”
9	”	”	”	”
28	SIV*env/rev* + SIV*gag*	Native SIVgp120 in MPL-SE	”	”
15	SIV*env/rev* + SIV*gag* + SIV$nef_{\Delta 1\text{-}19}$	”	”	”
33	”	”	”	”
41	”	”	”	”
4	SIV*env/rev* + SIV$nef_{\Delta 1\text{-}19}$	”	”	”
29	”	”	”	”
32	”	”	”	”
40	”	”	”	”
Mock-immunized control macaques for 1st challenge				
34	Ad5hr vector only	MPL-SE only	SIV_{mac251}	SIV_{mac251}
39	”	”	”	”
Naïve Macaques for 2nd challenge				
160	None	None	None	SIV_{mac251}
173	”	”	”	

[a] Each Ad5hr-SIV recombinant was administered at a dose of 5×10^8 pfu. The total Ad5hr dose was made up to 1.5×10^9 with the empty Ad5hr vector when the immunization mixture contained fewer than three recombinants. The week 0 immunization was intranasal; the week 12 intratracheal.

[b] Envelope subunits (100 μg/dose) were administered intramuscularly.

[c] The second IR challenge was given 53 weeks after the first with no intervening immunization.

(39%) immunized macaques. The immunogens these macaques received are summarized in Table 1. The strong protection in the 12 macaques following the first intrarectal SIV_{mac251} challenge was defined by their viral burdens, determined at three levels of sensitivity as detailed in Materials and methods: a routine nucleic acid sequence-based assay (NASBA) with a threshold level of <2000 SIV RNA copies/100 μl plasma; an intermediate assay with a detection limit of <500 copies/0.5 to 1 ml plasma, and a real-time assay with a sensitivity of <50 copies/0.5 to 1 ml plasma. As detailed in our previous report, four macaques had no detectable plasma viremia at the intermediate level (Fig. 1A) for the entire 40-week observation period, although SIV proviral DNA was detected on at least one occasion in plasma of each macaque post-challenge (Patterson et al., 2004). Four macaques cleared viremia below the intermediate level or strongly controlled viremia at the threshold level of detection by 8 weeks post-challenge (Fig. 1B). Four others controlled viremia by 8 to 12 weeks post-challenge with repeated fluctuations around the detection threshold (Fig. 1C). In contrast, eight mock-immunized control macaques exhibited high viral burdens post-challenge, except for two whose viremia declined to the detection threshold by 40 weeks post-challenge (Fig. 1D).

Protection in the immunized macaques compared to the controls was also evident by their CD4 counts in peripheral blood. Following the first intrarectal challenge, all 12 immunized macaques maintained CD4 counts above 200/mm^3 (Figs. 2A–C). In contrast, the mock-immunized controls displayed gradual depletion of CD4 cells to below 200/mm^3 except for macaque #39 that exhibited gradual viremia control and maintained CD4 cell counts above 200 until after the re-challenge (Fig. 2D). Three of the mock-immunized controls were euthanized due to AIDS within a year of the first challenge. Three others were lost to follow-up after transfer to another investigator for therapy studies. Macaques #34 and #39 were maintained for further study.

To evaluate the durability of protection, one year (week 53) after the first SIV_{mac251} intrarectal challenge, eleven of the original strongly protected vaccinees were re-challenged rectally with the same SIV_{mac251} stock. Mock-immunized control macaques #34 and #39 were also re-challenged in order to compare responses of the vaccinees with macaques that exhibited some natural control of viremia. Two naïve macaques served as controls for the challenge stock. Administration of the second SIV_{mac251} rectal challenge resulted in high viral burdens in the naïve macaques #160 and #173 (Fig. 1E). Acute viremia rose to over 1×10^8 SIV RNA copies/ml of plasma. At week 20, macaque #160 died of AIDS-related complications while the CD4 counts of macaque 173 dropped below 200/mm^3 (Fig. 2E). The unimmunized, chronically SIV-infected monkeys, #34 and #39, exhibited viremia around the detection threshold after the second SIV_{mac251} challenge (Fig. 1D), while their CD4 cells remained at or below 200/mm^3 (Fig. 2D).

Following administration of the second SIV_{mac251} challenge, three of the eleven previously protected monkeys exhibited sustained viremia above the detection threshold: #41, #4, and #40 (Figs. 1A, C), although up to a 3 log decrease in viral load

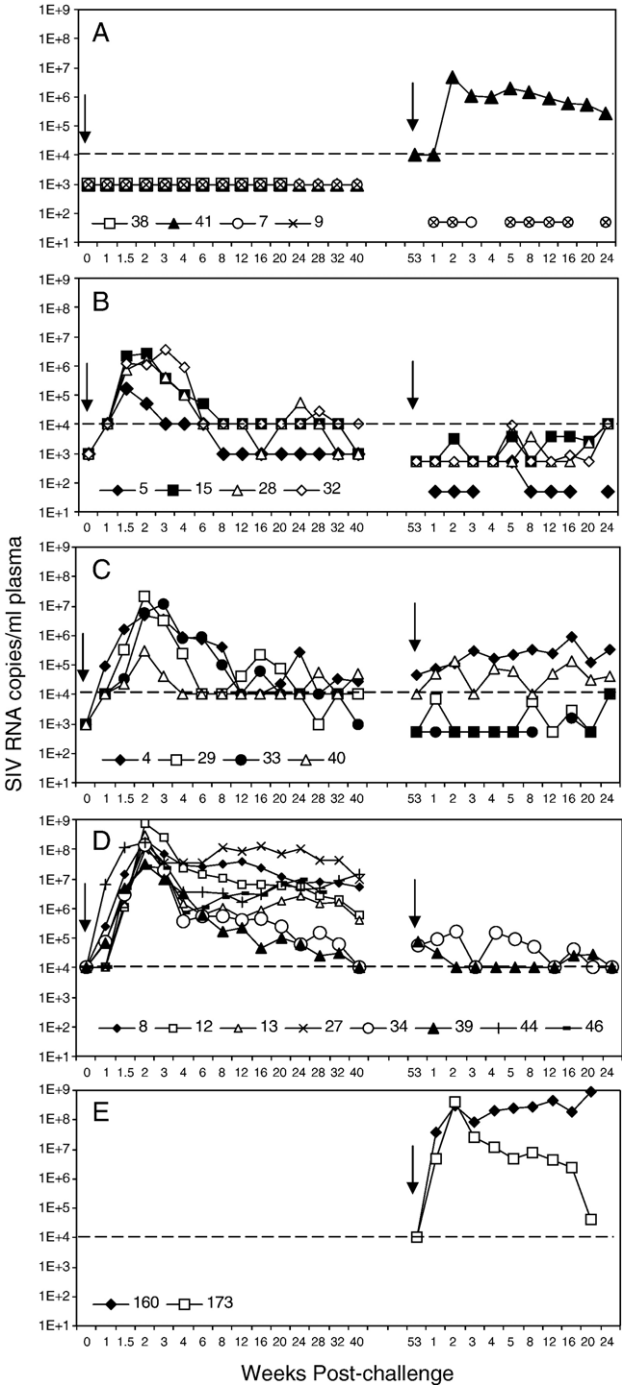

Fig. 1. Viral burdens in immunized and control macaques following rectal SIV_{mac251} challenges at weeks 0 and 53. (A) Immunized macaques exhibiting no detectable viremia following the first challenge. (B) Immunized macaques that cleared viremia or strongly controlled viremia at the threshold of detection following the first challenge. (C) Immunized macaques that controlled viremia with fluctuations around the detection threshold following the first challenge. (D) Control macaques from the first challenge experiment including two (#34 and #39) that exhibited natural control by week 40 and were subsequently re-challenged. (E) Naïve control macaques for the second SIV_{mac251} challenge. Viral burdens over weeks 0 to 40 were taken from Patterson et al. (2004). Arrows mark the SIV_{mac251} challenges at weeks 0 and 53. SIV RNA copies <2000/100 μl, representing the initial detection threshold, are plotted as 10,000 copies/ml (marked by the dashed line). Undetectable SIV RNA at the intermediate sensitivity of <500 copies/input volume (1 or 0.5 ml) is plotted as 500 or 10_3 copies/ml respectively. Undetectable SIV RNA at <50 copies/input volume is plotted as 50 copies/ml.

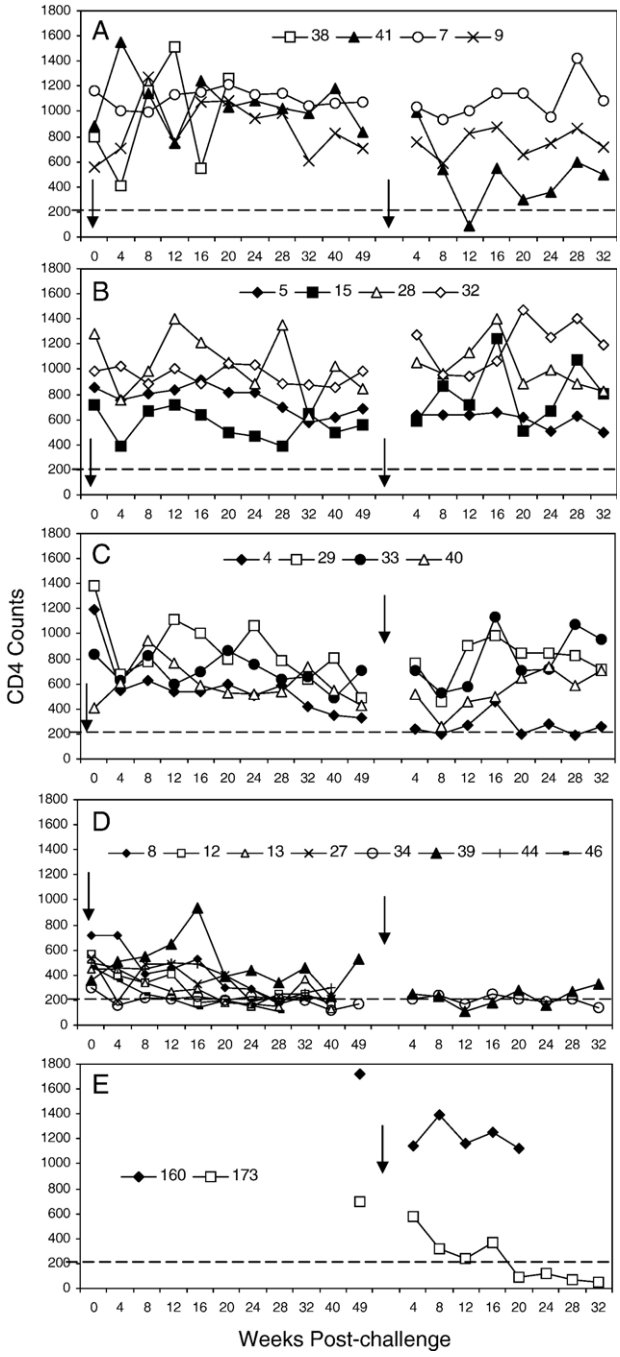

Fig. 2. Peripheral blood CD4+ T cell counts in immunized and control macaques following rectal SIV_{mac251} challenges at weeks 0 and 53. (A) Immunized macaques exhibiting no detectable viremia following the first challenge. (B) Immunized macaques that cleared viremia or strongly controlled viremia at the threshold of detection following the first challenge. (C) Immunized macaques that controlled viremia with fluctuations around the detection threshold following the first challenge. (D) Control macaques from the first challenge experiment including two (#34 and #39) that exhibited natural control by week 40 and were subsequently re-challenged. (E) Naïve control macaques for the second SIV_{mac251} challenge. Arrows mark the SIV_{mac251} challenges at weeks 0 and 53. The dashed line is placed at the level of 200 CD4+ T cells/mm^3 blood.

was observed compared to the naïve macaques (Fig. 1E). Further, only one of these three, macaque #4, exhibited a sustained drop in CD4 cell numbers to approximately 200/mm^3 (Fig. 2C), while macaques #40 and #41 exhibited only a transient decline in CD4 cell number which then rebounded (Figs. 2A, C).

In contrast to the modest viremia seen in these 3 immunized macaques, the eight other previously protected vaccinees remained strongly protected after the second challenge, with viral loads occasionally fluctuating above 500 SIV RNA copies/ml, but always remaining <20,000 copies/ml plasma (Figs. 1A–C). Three of these macaques, #5, #7, and #9, exhibited no plasma viral RNA at all in the intermediate assay, and therefore, where available, their plasma was assessed in the real-time assay with the greatest detection sensitivity. Macaques #7 and #9 again consistently exhibited undetectable viremia (<50 SIV RNA copies; Fig. 1A), as did macaque #5 except for a single time point with SIV RNA at the 500 copy level (Fig. 1B). In accordance with the strong control of SIV replication, all eight durably protected macaques maintained strong preservation of CD4+ T cell numbers (Figs. 2A–C). Overall, ten of eleven of the original strongly protected, immunized macaques maintained CD4 T cell counts >200/mm^3.

Cellular immune responses prior to re-challenge correlate with viremia control in durably protected macaques

To investigate a possible correlation between vaccine-induced immunity and control of viremia in the durably protected macaques, we performed a systematic evaluation of SIV-specific immune responses in all animals before and after the second SIV_{mac251} challenge, beginning with cellular immunity. Secretion of IFN-γ by PBMC in response to Env, Gag, Nef, and Rev peptide pools was evaluated by ELISPOT. Thirty days prior to the second challenge the protected group of eight macaques demonstrated a higher number of IFN-γ-secreting cells across all four peptides compared to immunized, viremic ($P = 0.037$), SIV-infected, unimmunized ($P = 0.05$), and naïve ($P = 0.01$) macaques (Table 2). The predominant response among the protected macaques was to Gag peptides, although Env peptides were well recognized in all but the naïve macaques. Following re-challenge, we did not observe a statistically significant difference in the number of IFN-γ-secreting cells to any of the peptide pools between the durably protected and any of the other macaque groups, although responses to Gag peptides in the protected group were somewhat higher at weeks 2, 5, and 8 (data not shown).

We also evaluated T cell proliferative responses to SIV gp120, Ald-SIV, SIV p27, and Nef before and after the second challenge (Table 2). Prior to the re-challenge, the protected group exhibited significantly higher proliferative responses to p27 compared to the immunized, viremic group ($P = 0.026$) but not compared to the SIV-infected unimmunized macaque #39. Similarly to the ELISPOT results, no significant differences were seen following re-challenge. Proliferative responses to gp120, Ald-SIV and Nef were generally low or negative, and no significant differences were observed among the groups of macaques before or after re-challenge.

Table 2
Cellular immune responses 30 days prior to SIV_{mac251} re-challenge

Macaque	ELISPOT (SFC/10^6 PBMC)				Proliferation (Stimulation index)			
	Gag	Env	Nef	Rev	gp120	Ald-SIV	p27	Nef
Naïve control								
160	0	0	0	0	nd	nd	nd	nd
173	0	0	0	0	nd	nd	nd	nd
Mean ± SEM	0	0	0	0				
Unimmunized, SIV-infected								
34	0	0	0	0	nd	nd	nd	nd
39	26	159	36	0	1.1	3.2	7.6	1.4
Mean ± SEM	13 ± 13	80 ± 80	18 ± 18	0	1.1	3.2	7.6	1.4
Durably protected								
5	574	68	0	0	1.4	4.4	1.9	0.3
7	38	0	0	0	2.6	0.7	2.0	0.8
9	336	52	177	0	0.8	0.3	0	0
15	48	0	0	0	2.7	0.03	7.9	1.1
28	57	13	87	0	4.1	0.04	22.3	1.9
29	572	0	51	0	1.7	0.03	0.04	3.4
32	286	0	0	0	2.7	0.03	7.9	1.1
33	0	321	0	0	3.9	0.01	23.9	0.5
Mean ± SEM	239 ± 85 [a]	57 ± 39 [a]	39 ± 23 [a]	0 [a]	2.4 ± 0.4	0.7 ± 0.5	7.3 ± 3.6 [b]	1.2 ± 0.4
Immunized, viremic								
4	0	0	0	0	1.3	0.06	0.1	1.2
40	0	433	38	35	3.0	0.03	0.8	0.5
41	23	0	0	0	3.1	0.7	0	0.5
Mean ± SEM	8 ± 8	144 ± 144	13 ± 13	12 ± 12	2.4 ± 0.6	0.3 ± 0.2	0.3 ± 0.2 [b]	0.7 ± 0.2

Macaques are grouped as durably protected (eight original immunized, protected macaques that exhibited durable protection following re-challenge); immunized, viremic (three immunized, previously protected macaques that became viremic following re-challenge); SIV-infected, unimmunized (two original unimmunized control macaques that gradually controlled viremia by week 40 after the first challenge); and naïve (two macaque controls for the SIV_{mac251} re-challenge).
nd = Not determined as viable PBMC were not available for the two naïve macaques nor for macaque #34 in the SIV-infected, unimmunized group.
[a] The durably protected macaques exhibited a higher number of IFN-γ-secreting cells across all four peptides compared to the immunized viremic ($P = 0.037$), SIV-infected, unimmunized ($P = 0.05$), and naïve ($P = 0.01$).
[b] The durably protected macaques exhibited higher proliferative responses to p27 compared to the immunized viremic macaques ($P = 0.026$).

Anti-SIV envelope antibodies do not correlate with protection against re-challenge

In our previous work, we demonstrated a significant negative correlation between acute viremia and serum binding antibodies to envelope following the initial intrarectal challenge with SIV_{mac251} (Patterson et al., 2004). These binding antibodies were subsequently shown to mediate ADCC activity that was significantly correlated with the reduced acute-phase viremia (Gómez-Román et al., 2005). Therefore, we examined anti-SIV envelope antibodies in serum and mucosal secretions in order to evaluate humoral immunity as a possible mechanism of durable protection. Table 3 summarizes the results of assays evaluating both binding antibodies and functional antibodies mediating neutralization and ADCC activity. In general, serum antibody responses did not correlate with protection against the SIV_{mac251} re-challenge. Two of the durably protected macaques, #7 and #9, exhibited little anti-envelope binding, neutralizing or ADCC antibodies at the time of re-challenge. These macaques had been boosted with the SIV peptomer and never developed strong antibodies to SIV gp120 (Patterson et al., 2004). As they also were aviremic following the initial SIV_{mac251} challenge, they did not develop anti-envelope antibodies attributable to SIV infection. Note that macaque #5, similarly immunized, did exhibit anti-envelope antibody responses, as a result of both the earlier Ad5hr-SIV*env/rev* priming immunizations and transient virus exposure following the initial challenge. Binding antibody titers were already high in all the remaining immunized and unimmunized SIV-infected macaques (Table 3); therefore anamnestic antibody responses following re-challenge were not observed except in macaque #41 (data not shown), reflecting the rapid infection and viremia increase in this macaque. Although neutralizing antibodies to T-cell-line-adapted SIV_{mac251} were present, none of the macaque sera neutralized primary SIV_{mac251} in an infectivity reduction assay. Antibodies mediating ADCC were readily detected in all but the naïve macaques and macaques #7 and #9, but again did not correlate with protective outcome post-re-challenge.

Mucosal anti-envelope binding antibodies were also evaluated in rectal secretions and saliva. SIV-specific IgA antibodies were not observed either before or after the SIV_{mac251} re-challenge (data not shown). Anti-SIVgp120 IgG antibodies

Table 3
Antibodies to SIV envelope at the time of re-challenge in sera and mucosal secretions

Macaque	Binding titer to SIV gp120	Neutralizing antibody titer[a]	ADCC mediating titer		Rectal IgG (O.D.)		Salivary IgG (O.D.)	
			H9/SIV targets	CEM.NKr/gp120 targets	Pre-re-challenge[b]	Anamnestic response[c]	Pre-re-challenge[b]	Anamnestic response[c]
Naïve control								
160	<10	<10	<100	<100	0.11	0.30	0.19	0.19
173	<10	25	100	100	0.13	0.17	0.14	0.29
Unimmunized, SIV-infected								
34	1.9×10^6	1.4×10^4	1×10^7	1×10^6	**0.65**	0.87	**1.25**	1.37
39	12.3×10^6	2.2×10^4	$>1 \times 10^7$	1×10^7	0.08	**1.24**	1.55	1.46
Durably protected								
5	1.7×10^5	3.2×10^3	1×10^5	1×10^5	0.08	0.09	0.31	0.50
7	7.5×10^1	<10	<100	<100	0.11	0.11	0.12	**0.53**
9	<10	<10	<100	<100	0.08	0.09	0.11	0.11
15	6.4×10^5	1.8×10^4	1×10^6	1×10^6	0.21	0.30	**0.58**	1.06
28	5.1×10^5	1.6×10^4	1×10^6	1×10^6	0.16	0.17	**1.10**	1.08
29	1.1×10^5	2.0×10^4	1×10^5	1×10^6	**0.26**	0.65	1.86	1.80
32	1.5×10^5	1.1×10^4	1×10^5	1×10^6	**0.30**	0.21	**1.12**	1.02
33	1.7×10^5	2.1×10^4	1×10^5	1×10^6	0.22	0.20	**1.11**	1.53
Immunized, viremic								
4	3.0×10^5	2.3×10^4	1×10^6	1×10^6	0.11	0.15	**0.74**	1.25
40	2.0×10^6	4.2×10^4	$>1 \times 10^7$	1×10^7	**1.32**	1.86	**1.98**	2.21
41	2.3×10^4	1.5×10^3	1×10^4	1×10^6	0.15	0.16	0.29	**0.64**

[a] Neutralizing antibody against TCLA-SIV_{mac251}. All sera were negative (<10-fold decrease in viral infectivity) for neutralization of primary SIV_{mac251} in an infectivity reduction assay.
[b] Positive IgG response in bold defined as equal to or greater than two times the mean O.D. of the naïve control macaques.
[c] Positive anamnestic response in bold defined as equal to or greater than two times the pre-re-challenge O.D. value.

were detected rarely prior to the re-challenge in rectal secretions, but more frequently in saliva (Table 3). This may reflect the greater dilution of the rectal samples tested (1:100) compared to the dilution of saliva (1:10). Anamnestic responses following the re-challenge were also rarely observed. Overall, there was no correlation of SIV-specific mucosal binding antibody with durability of protection.

In vivo CD8+ T lymphocyte depletion of durably protected macaques

Evaluations of immune responses following the re-challenge suggested an important role of SIV-specific CD8+ T lymphocytes in conferring durable protection against pathogenic SIV_{mac251}. Therefore, 9 months after the second challenge, we depleted CD8+ T cells in vivo from the eight durably protected macaques and one SIV-infected unimmunized monkey, #39, in order to confirm the involvement of CD8+ T cells in containment of SIV replication. An additional SIV-infected unimmunized monkey, #34, was given an IgG isotype control antibody in order to demonstrate that any subsequent change in viremia level or immune response was due to CD8+ T cell depletion rather than to IgG itself.

Administration of 3 injections of antibody cM-T807 to macaques depleted 97–99% of CD8+ T cells from peripheral blood for a period of about 10 days (Fig. 3). Concomitant with CD8 depletion, the macaques exhibited a rapid emergence of viremia, ranging from 10^5 to 10^8 SIV RNA copies/ml plasma (Fig. 3). As CD8+ T cells reappeared in the blood around day 21, viral replication was brought under control. Of interest, CD8 depletion in macaques #7 and #9 was not associated with emergence of viremia. In fact, at all time points, viremia in these macaques was <50 SIV RNA copies/ml plasma (Fig. 3). The subset of CD4+ T cells was relatively stable during the course of CD8 depletion (data not shown). A transient reduction was observed at day 3 post-depletion. Thereafter, CD4 counts remained at predepletion levels until approximately day 21 when counts exhibited a modest rise as viremia was controlled. The SIV-infected, unimmunized macaque #39 showed elevated viremia during CD8 depletion, however, in contrast to the durably protected macaques, when CD8+ T cells were restored the viral load was not contained (Fig. 3). Macaque #34 exhibited slight fluctuations of CD8+ T cells and viral load after IgG isotype administration (Fig. 3). Such fluctuations are not unusual, as has been described by others (Schmitz et al., 1999a).

Because SIV infects tissue lymphocytes as well as PBL, we monitored CD8+ T cell levels and the level of SIV RNA in lymph node (LN) and rectal tissue biopsies before and after administration of the cM-T807 antibody. Thirty days prior to depletion of CD8+ T cells SIV RNA was not detected in rectal tissues of either the protected macaques or the chronically SIV-infected monkeys (Table 4) although adequate target cells for viral infection were present. CD3+/CD4+ T cells in rectal tissue

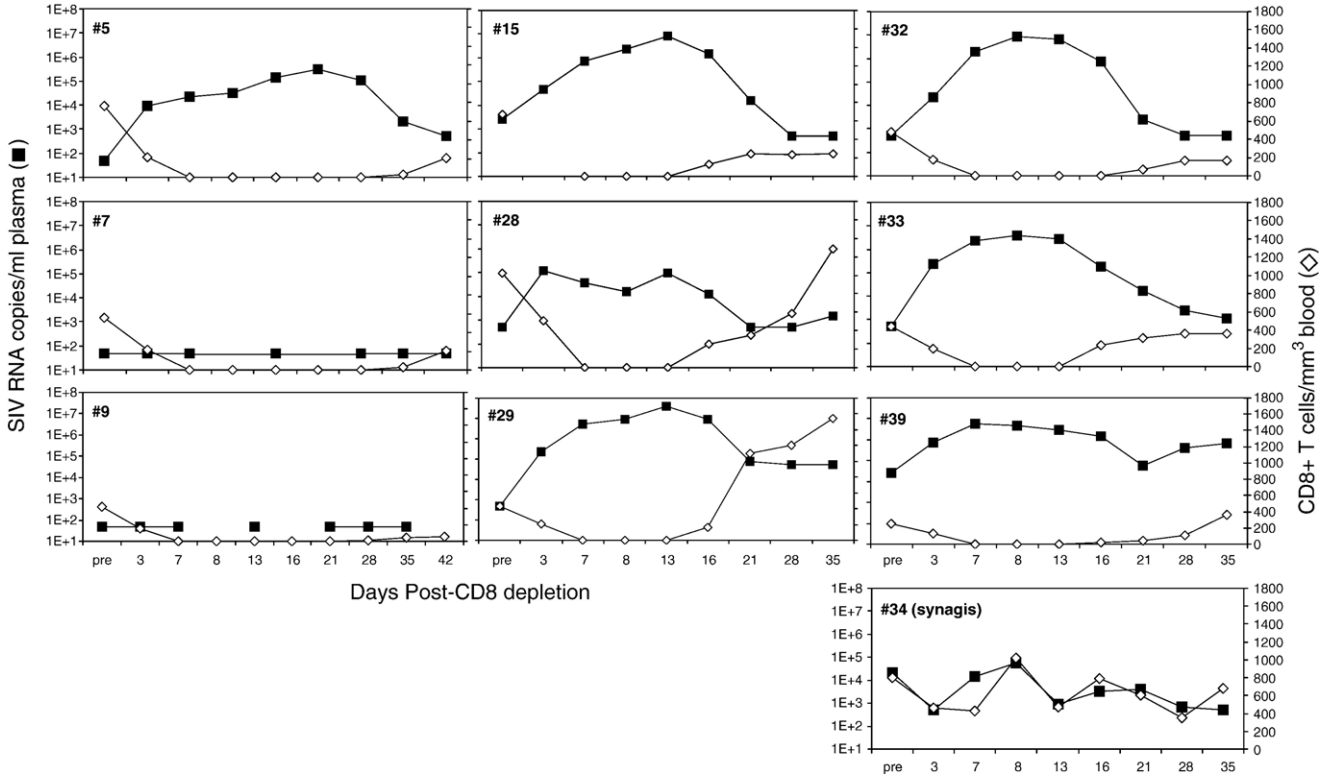

Fig. 3. In vivo depletion of CD8+ T cells and associated changes in SIV plasma viremia. The 8 durably protected macaques (numbers 5, 7, 9, 15, 28, 29, 32, and 33) are presented in separate panels, along with one unimmunized, SIV-infected macaque (#39), and one unimmunized, SIV-infected macaque (#34) that received the isotype control antibody, synagis. The cM-T807 antibody or control antibody was administered to the macaques on days 0, 3, and 7, as described in Materials and methods.

before administration of the anti-CD8 antibody were 19.3% and 28.2% in the unimmunized, SIV-infected macaques #34 and #39, respectively, and ranged from 29.1% to 68.9% in the durably protected macaques. Lymph node CD3+/CD4+ T cells prior to CD8+ T cell depletion were 47.3% and 44.3% in macaques #34 and #39, respectively, and ranged from 47.7% to 67.9% in the durably protected animals. After administration of anti-CD8 antibody, on average only 37% of CD8 T cells were depleted from rectal tissue. It has been shown, however, that binding of cM-T807 to CD8+ T cells diminishes their function (Schmitz et al., 1999b), so loss of CD8+ T cell activity might have been greater than suggested by the level of depletion. Overall, the antibody treatment resulted in emergence of detectable virus in rectal samples from the unimmunized, SIV-infected macaque, #39, and from all the durably protected macaques except #7 and #9. Viral burdens ranged from 1×10^4 to 4×10^6. Virus did not emerge in the unimmunized SIV-infected macaque (#34) treated with the control synagis antibody. With restoration of CD8 T cells, all macaques but two regained viremia control. The two exceptions, #5 and #33, had exhibited the highest viral burdens in rectal tissue at day 13 ($>10^6$ copies/μg RNA) but were able to reduce the viral load 3 logs by day 64.

In contrast to rectal tissue, administration of the anti-CD8 antibody depleted a higher proportion of CD8+ T cells from LN, on average 87%. Unfortunately, analysis of SIV RNA in LN was not possible in all macaques, as sufficient RNA could not always be extracted from the biopsies. Of the samples analyzed, 4 of 6 durably protected macaques and the two unimmunized, SIV-infected macaques exhibited SIV RNA prior to depletion, with somewhat lower copy numbers seen in the protected animals (Table 4). At the time of peak CD8 depletion, viral burdens were elevated in the LN biopsies of three of the four protected macaques able to be analyzed. However, viral loads were not significantly decreased with restoration of T cell numbers but remained similar to levels seen prior to or during CD8 depletion. Overall, CD8 depletion and restoration did not coincide with emergence and control of virus in LN as was seen in rectal tissue. Of interest, tissues of macaques #7 and #9 were negative for SIV RNA at all time points that could be analyzed.

Anti-SIV immunity during CD8 depletion

ELISPOT responses in PBMC to Env, Gag, Nef and Rev peptide pools were evaluated prior to, during, and after CD8 depletion. Before depletion, the durably protected macaques demonstrated a dominant cellular response to Env peptides and modest responses to Gag, Nef, and Rev peptide pools (Fig. 4A). Between days 7 and 13, the time of greatest CD8+ T cell depletion, responses to Env, Nef, and Rev were markedly decreased. Responses to Gag peptides, however, showed little change over time, perhaps reflecting significant numbers of SIV Gag-specific CD4+ T cells in the PBMC. To evaluate this

Table 4
Viral loads and level of CD8+ T cells in rectal tissue and lymph nodes before and after CD8 depletion

Tissue	Macaque	SIV copies/μg RNA			%CD3+/CD8+ T cells		
		−30 days	Day 13	Day 64	−30 days	Day 13	Day 64
Rectal tissue	Durably protected						
	#5	<500	1.0×10^6	3.6×10^3	46.7	8.5	28.1
	#7	<500	<500	<500	30.5	12.3	24.8
	#9	<500	<500	<500	58.1	21.4	30.0
	#15	<500	1.3×10^4	<500	73.1	52.0	43.3
	#28	<500	3.1×10^4	<500	56.7	37.8	34.2
	#29	<500	5.8×10^5	<500	60.5	49.3	50.6
	#32	<500	5.0×10^5	<500	52.0	50.7	30.0
	#33	<500	4.0×10^6	1.4×10^3	42.3	37.2	40.0
	Unimmunized, SIV infected						
	#39	<500	8.6×10^3	<500	70.8	47.6	57.4
	#34 (Synagis)	<500	<500	<500	79.4	88.4	57.3
Lymph node	Durably protected						
	#5	3.9×10^4	nd	4.1×10^5	31.3	3.3	14.9
	#7	nd	nd	<500	31.4	1.4	9.6
	#9	<500	<500	<500	19.3	0.7	7.6
	#15	nd	nd	7.4×10^4	32.6	11.8	28.6
	#28	<500	7.4×10^3	1.3×10^5	30.2	7.9	26.2
	#29	9.8×10^4	1.0×10^6	2.1×10^5	28.8	0.8	19.2
	#32	2.9×10^4	nd	2.1×10^4	24.8	2.9	16.8
	#33	5.0×10^4	2.0×10^5	nd	32.0	2.8	17.1
	Unimmunized, SIV infected						
	#39	2.1×10^5	6.8×10^5	5.5×10^5	35.0	5.5	26.8
	#34 (Synagis)	3.2×10^5	8.4×10^4	1.3×10^5	32.8	28.4	53.7

nd = Not done, as insufficient RNA was extracted from the biopsied samples.

possibility, we subsequently performed an ELISPOT analysis on cells obtained 38 to 41 weeks following in vivo CD8 depletion from two macaques, #29 and#33, using unfractionated PBMC and PBMC depleted of either CD4 or CD8 cells in vitro. Both macaques exhibited abundant numbers of IFN-γ-secreting cells when CD8 cells were depleted but decreased numbers of IFN-γ-secreting cells (approximately 36% of the unfractionated PBMC response) when CD4 cells were depleted (data not shown). Although we do not know that this CD4 response was also present during the time of the greatest CD8 depletion, the result obtained suggests the persistent presence of SIV Gag-specific CD4+ T cells in at least these two macaques. Overall, the re-appearance of CD8+ T lymphocytes and containment of viremia beginning at day 21 post in vivo CD8 depletion coincided with higher numbers of IFN-γ-secreting cells to Env, Gag, Nef, and Rev peptides (Fig. 4A).

In contrast to the immunized, durably protected macaques, the SIV-infected, unimmunized macaque #39 exhibited many fewer IFN-γ-secreting cells in response to Env, Gag, Nef, and Rev peptides over the course of CD8 depletion (Fig. 4B). This result illustrates a difference in level of immune response of the durably protected macaques that exhibit potent, persistent immunity elicited by the vaccine regimen in comparison to that induced by natural SIV infection, although a firm conclusion cannot be made based on this single animal.

We also measured antigen-specific lymphoproliferative responses of PBMC to gp120, Ald-SIV, p27, and Nef proteins before and after CD8 depletion. Thirty days prior to depletion, the protected group exhibited strong proliferative responses to gp120, Ald-SIV, and p27 (Fig. 4C). These responses fluctuated but were maintained throughout the CD8 depletion period. In contrast, the SIV-infected, unimmunized macaque, #39, had diminished CD4+ T cell counts (see Fig. 2D) and proliferative responses remained low at all time points (Fig. 4D). These findings again suggest a vaccine effect on the persistence of anti-SIV immunity, although evaluation of more SIV-infected unimmunized macaques would be needed to prove the point.

It was also of interest to evaluate immune responses of cells in tissue compartments before and at days 13 and 64 after depletion. Because cell numbers were limited, we performed IFN-γ ICS on fresh cells from LN biopsies and rectal pinches in response to mixed SIV Env, Gag, and Nef peptide stimulation (Fig. 5). Thirty days prior to depletion, immune responses were not detected in LN, however, at day 13 in spite of the overall 87% depletion of CD8+ lymphocytes, IFN-γ-secreting cells increased to 0.25% of the remaining CD8+ T cells, suggesting a rapid response to the emergence of virus. These cells persisted and underwent further expansion by day 64 when the SIV-specific IFN-γ-secreting CD8+ T cell population reached 1.3%. Rectal tissue already exhibited a positive IFN-γ response to the mixed peptide stimuli prior to CD8 depletion. CD8 T cell numbers sufficient for analysis were not recovered at day 13 following administration of the cM-T807 antibody. However, as the CD8 T cell population was restored, SIV-specific IFN-γ-secreting cells reappeared robustly to a level of 3.8% (Fig. 5). This increased response at day 64 post-depletion in rectal tissue was associated with containment of localized virus (Table 4).

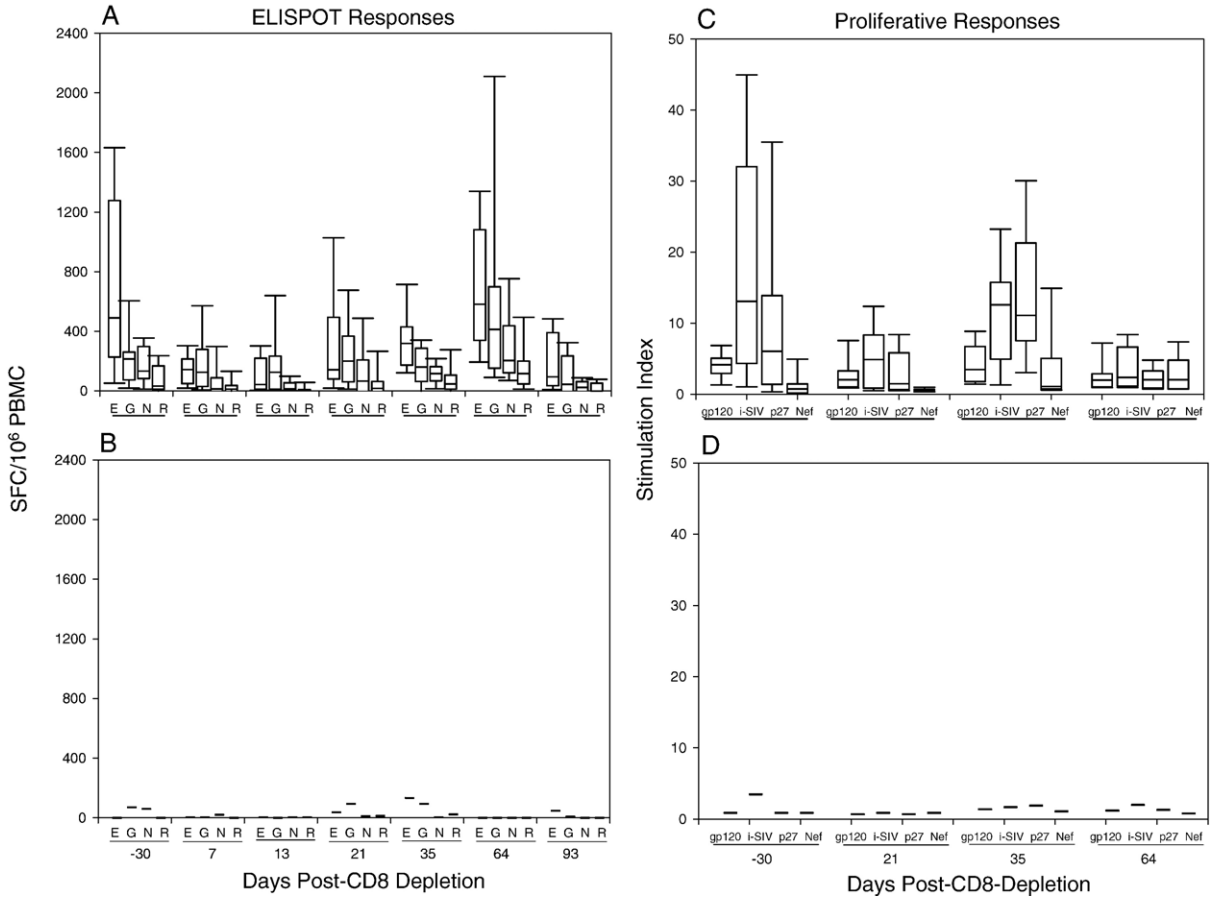

Fig. 4. Cellular immune responses observed before and after in vivo CD8 depletion. ELISPOT responses to SIV peptide pools over the course of depletion are shown for the durably protected macaques (A) and the unimmunized, SIV-infected macaque #39 (B). The peptide pools are indicated as E, G, N, and R, representing Env, Gag, Nef, and Rev, respectively. T cell proliferation responses to SIV proteins are shown in the durably protected macaques (C) and the unimmunized, SIV-infected macaque #39 (D) before and after CD8 depletion. The antigenic stimuli for the proliferative responses are indicated under each box plot. i-SIV = aldrithiol-inactivated SIV. The box plots include median values and 5 and 95% percentiles for the durably protected macaques.

In vitro infectivity and proviral DNA in strongly protected macaques #7 and #9

From the time of initial SIV_{mac251} infection at day 0, throughout re-challenge with SIV_{mac251} at week 53 and subsequent in vivo CD8 depletion, durably protected macaques #7 and #9 never showed any detectable viremia, even following the effective depletion of CD8+ T lymphocytes. We previously reported that these macaques exhibited occasional provirus positivity, indicating that they did not have "sterilizing" immunity (Patterson et al., 2004). To elucidate possible immunologic mechanisms associated with their remarkably strong, durable protection, a detailed summary of their immune responses pre- and post-re-challenge and CD8 depletion is presented in Table 5. Macaque #7 exhibited positive proliferative responses to both SIV Gag and envelope proteins before and after the SIV_{mac251} re-challenge, respectively, as well as a stronger proliferative response to SIV gp120 and a potent response to SIVp27 before in vivo CD8 depletion that may have contributed to the protective outcome. While the ELISPOT responses of macaque #7 were low or undetectable at the times of re-challenge and in vivo CD8 depletion, the numbers of IFN-γ-secreting cells in response to Env peptide stimulation were increased after the re-challenge, and responses to all 4 peptide pools were elevated following CD8 depletion. These results suggest not only the presence of memory cells able to respond to the challenge virus but also

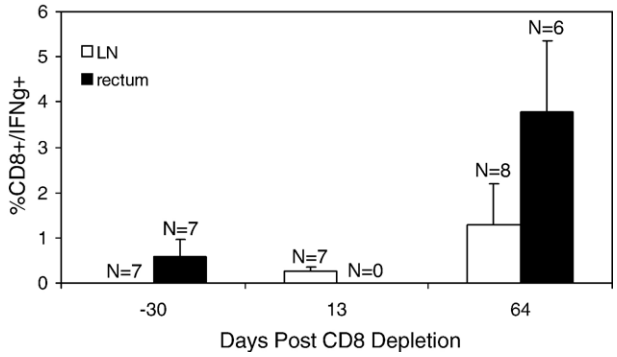

Fig. 5. Intracellular staining of CD8+ IFN-γ+ T cells in lymph nodes and rectal tissue in response to stimulation with a mixture of SIV Env, Gag, and Nef peptides before and after CD8 depletion. The numbers of tissue biopsies tested are indicated above each bar. At day 13 post-depletion, the number of CD8 T cells in rectal tissue was insufficient for analysis.

Table 5
SIV-specific immune responses of macaques #7 and #9 pre- and post-re-challenge and CD8 depletion

	ELISPOT (SFC/10^6 PBMC)				T cell proliferation (Stimulation index)			Env binding antibodies		
	Env	Gag	Nef	Rev	gp120	Ald-SIV	p27	Serum (Titer)	Rectal IgG/IgA	Salivary IgG/IgA
Macaque #7										
Pre-re-challenge [a]	0	38	0	0	2.6	neg	2.0	75	neg	neg
Post-re-challenge [b]	31	0	0	0	3.5	3.3	2.0	115	neg	Pos IgG
Pre-CD8 depletion [c]	0	0	0	0	4.1	neg	18.2	neg	nd	nd
Post-CD8 depletion [b]	230	17	135	235	neg	neg	neg	neg	nd	nd
Macaque #9										
Pre-re-challenge [a]	52	336	177	0	neg	neg	neg	neg	neg	neg
Post-re-challenge [b]	314	224	80	20	neg	2.0	neg	165	neg	neg
Pre-CD8 depletion [c]	336	12	103	0	neg	neg	2.1	75	nd	nd
Post-CD8 depletion [b]	621	577	136	62	5.4	neg	8.9	75	nd	nd

Pos IgG = positive for IgG antibody.
[a] Responses were evaluated 30 days before re-challenge.
[b] Peak responses following re-challenge over weeks 1 to16 and CD8 depletion over days 7 to 93 are recorded.
[c] Responses were evaluated 30 days before CD8 depletion.

suggest the presence of a sequestered SIV reservoir, activated when CD8 cells were depleted, and to which the macaque responded. In contrast to macaque #7, macaque #9 exhibited relatively poor proliferative responses but strong ELISPOT responses to the SIV peptide pools. The absence of significant mucosal antibody responses to SIV envelope and detection of only low level serum anti-envelope binding antibodies suggest a strong role played by cellular immune responses in controlling viral infection in these macaques. It must be pointed out, however, that boosting with the SIV peptomer may have elicited antibodies recognizing unique conformational epitopes of the viral envelope not represented in the monomeric gp120 used in the ELISA evaluations. The possibility that such antibodies exist is undergoing further investigation.

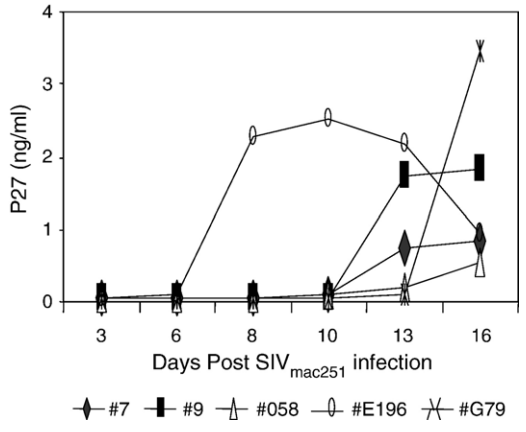

Fig. 6. In vitro SIV_{mac251} infection of PBMC from macaques #7, #9 obtained prior to immunization and of naïve macaques, #058, #E196, and #G79. Supernatants were harvested from the cultures at the indicated days and SIV p27 was quantified by antigen capture assay. Results from the last limiting viral dilution (1:125) at which cells of all macaques were infected are plotted. The SIV_{mac251} stock used for infection contained 17.5 ng/ml p27.

The strong protection of macaques #7 and #9 against the highly virulent SIV_{mac251}, manifested by undetectable viremia in peripheral blood, LN and rectal tissue, suggested that they might be resistant to SIV infection. Mamu-A*01 and Mamu-B*17 positive macaques have been shown to be more resistant to SIV infection compared to macaques of other MHC class I types (Muhl et al., 2002; O'Connor et al., 2003; Pal et al., 2002), however, neither macaque #7 nor #9 was positive for either of these alleles. Nevertheless, other host resistance factors might be operative in these animals. To address this question, we assessed the ability of viably frozen PBMC of macaques #7 and #9 obtained prior to Ad5hr-SIV recombinant immunization to be infected by SIV in vitro. The PBMC of 3 naïve macaques, used as positive controls, were readily infected by SIV_{mac251} (Fig. 6). The PBMC of macaques #7 and #9 exhibited productive infection with slightly delayed kinetics compared to naïve control #E196, but their rate and level of infection were comparable to the other controls, #058 and #G79. Thus, if macaques #7 and #9 are unusually resistant to SIV infection, it must be attributable to an in vivo mechanism rather than to innate host resistance.

Discussion

An effective AIDS vaccine will need to protect against multiple HIV exposures. In the present long-term study, we demonstrate for the first time remarkably strong protection against two sequential pathogenic SIV_{mac251} rectal challenges in macaques primed with replicating Ad5hr-SIV recombinant vaccines and boosted with SIV envelope subunits. Previously, such strong protection was achieved only by live-attenuated SIV vaccination (Connor et al., 1998; Daniel et al., 1992). In the first phase of this experiment, following an initial SIV_{mac251} intrarectal challenge 39% (12/31) of immunized macaques exhibited potent protection (Patterson et al., 2004). Now we report durable protection in 73% (8/11) of this earlier subset after

a second SIV_{mac251} challenge 1 year later with no intervening immunizations.

Immune mechanisms of this durable protection were investigated. Prior to the re-challenge, ELISPOT responses across the SIV peptide pools evaluated were significantly elevated in the immunized, durably protected macaques compared to the other macaques studied. In addition, the durably protected monkeys exhibited a significantly elevated lymphoproliferative response to Gag before the re-challenge compared to macaques in the other groups. Thus we conclude that the durable protection was associated with potent anti-SIV cellular immune responses. These results are in concert with our previous findings that correlated reduced viremia at set point with SIV Env- and Rev-specific cellular immune responses prior to the first SIV_{mac251} challenge (Patterson et al., 2004). Yet, this cellular immunity was also in part attributable to the undetectable or low-level persistent SIV infection established following initial SIV_{mac251} challenge in the protected macaques that quickly controlled viremia. For example, cellular responses to Gag were especially prominent at the time of re-challenge, yet not all the animals were immunized with the Ad5hr-SIV*gag* recombinant (Table 1).

In contrast to the association with cellular immunity, there was no correlation of binding, neutralizing, or ADCC-mediating antibody responses with protective outcome following the re-challenge. In fact, antibodies able to neutralize primary SIV_{mac251} were not detected in any of the macaques. In general, the SIV-infected, unimmunized macaques exhibited binding and ADCC-mediating antibodies with somewhat higher titer compared to the immunized macaques, reflecting the higher viral loads in these animals.

To confirm the role of SIV-specific CD8+ T cells in protection against SIV_{mac251}, the eight durably protected vaccinees were depleted of CD8 T cells in vivo. The coincident increase in viral load with CD8 depletion in all macaques except #7 and #9 agrees with similar reports illustrating the role of CD8 T cells in controlling viral replication in infected macaques and following immunization and challenge with SIV or SHIV isolates (Jin et al., 1999; Matano et al., 1998; Metzner et al., 2000; Schmitz et al., 1999a). The re-appearance of CD8+ T cells suppressed viral loads and enhanced IFN-γ secretion to all SIV antigens in the eight durably protected macaques but not in the SIV chronically infected macaque #39. Throughout the experiment unlike the durably protected macaques, macaque #39 never exhibited cellular immune responses to SIV antigenic stimulation. Although statistical analysis is not possible with only a single control macaque, this result suggests that the vaccine regimen may have contributed to the sustained, potent T cell responses that effectively contained SIV_{mac251}. This suggestion remains to be further investigated.

In these experiments, while we focused on CD3+/CD8+ T cells, the anti-CD8 monoclonal antibody used will also deplete those NK and NKT cells which are CD8+ (Motsinger et al., 2003; Sandberg et al., 2002). Although control of HIV/SIV replication mediated by these cells has not been established, both may contribute to protective responses. NK cells have been implicated in the protection of individuals who are highly exposed to HIV but remain uninfected (Scott-Algara et al., 2003). NKT cells contribute to protective immunity against a variety of pathogens, primarily through their regulatory role in amplifying initial innate immunity signals, thus promoting both Th1 and Th2 responses (Taniguchi et al., 2003). Therefore, we cannot not categorically conclude that protection was mediated only by CD8+ T cells. The contribution of NK and NKT cells in control of HIV/SIV infection remains to be elucidated.

CD8 depletion by the anti-CD8 monoclonal antibody was not as effective in tissues as in peripheral blood where nearly complete depletion was achieved. Approximately 37% of rectal CD8 cells were depleted, and about 87% of LN CD8 cells, a bit less than the 90 to 100% depletion reported in other studies (Schmitz et al., 1999a, 1999b). LN CD8 depletion was associated with slightly elevated virus loads and immune responses, however, with restoration of CD8+ T cells, viral burdens did not decline. The reason for lack of viral containment is not known. It has been reported that during chronic SIV infection the proportion of naïve CD4+ T cells and proliferative CD4+CCR5+ memory T cells present in tissues is critical with regard to the ability of the host to control viremia (Picker et al., 2004). A more complete characterization of LN cell subsets present during the in vivo depletion might have been informative with regard to this issue.

Surprisingly in view of the known propensity for SIV to infect CD4+CCR5+ T cells in the intestinal mucosa, while LN were SIV positive, no SIV RNA was detected in rectal tissue of any of the macaques prior to CD8 depletion, including the SIV chronically infected unimmunized macaque #39. The rectal biopsy samples, taken 1 to 3 in. from the anus, may not have adequately represented the entire gastrointestinal (GI) tract. SIV is known to rapidly infect the intestinal mucosa (Li et al., 2005; Mattapallil et al., 2005). SIV replication might be more readily detected in biopsies of the upper GI tract obtained by endoscopy, a procedure with some risk that can jeopardize further studies. Evaluation of rectal pinch biopsies of highly SIV-infected macaques could address whether the rectal sites sampled appropriately mirror infection of the upper GI tract. As recently reported, intestinal infection can be very localized (Li et al., 2005; Miller et al., 2005). While virus can be easily detected in peripheral LN during acute infection, small, discrete founder populations at the mucosal portal of entry drive virus replication and dissemination. Overall, in vivo CD8 depletion was associated with marked increases in both viral load and SIV-specific CD8+ T cell responses in rectal tissue. Importantly, with restoration of CD8+ cells, control of SIV was regained. Therefore, in the highly protected macaques, CD8+ T cell responses, perhaps including those of NK and NKT cells as discussed above, may have contributed to local containment of the virus at the rectal site.

The potent protection of macaques #7 and #9 at the level of continuous, undetectable virus in PBMC, LN, and rectal tissue, was most likely due to host immune responses, rather than genetic factors of resistance. This conclusion is based on the persistent cellular immune responses of the two macaques, and the susceptibility to in vitro infection by SIV_{mac251} of PBMC

obtained from the macaques prior to any immunization or challenge. Further, as noted above, macaques #7 and #9 are negative for both the Mamu-A*01 and Mamu-B*17 alleles associated with greater resistance to SIV infection. It was recently reported that Mamu-A*01 negative macaques rely less on CD8+ lymphocytes for viremia control compared to Mamu-A*01 positive macaques, suggesting a greater role of other immune mechanisms such as humoral immunity in containing viremia (Schmitz et al., 2005). However, we were able to detect only low-level anti-envelope binding antibodies in these animals, and functional antibodies were not observed. The elevated ELISPOT and/or lymphoproliferative responses observed in PBMC of macaques #7 and #9 following re-challenge and/or CD8 depletion despite the absence of detectable viremia suggest on-going, low-level, but anatomically contained SIV replication. Similar viremia control at undetectable levels resulting from vaccination followed by low-level infection with $SHIV_{IIIB}$ or by SIV infection followed by anti-retroviral therapy has been attributed to cellular immunity induced or maintained by inapparent viral replication (Kim et al., 2001; Lifson et al., 2000, 2001). Such control of $SIV_{\Delta B670}$ replication following vaccination has been attributed to low-level viral replication in a reservoir located in the gastric-associated lymphoid tissue (Fuller et al., 2002). In experiments with live attenuated SIV, it was similarly reported that minimal, undetectable viral replication might have been sufficient to provide antigenic stimulation and sustain potent anti-viral cellular immunity (Connor et al., 1998). Analysis of other anatomical compartments of macaques #7 and #9 may reveal viral reservoirs that were not detected in peripheral blood, inguinal lymph nodes, or rectal tissue.

Following vaccination and the initial SIV_{mac251} intrarectal challenge, the 11 macaques studied here exhibited potent protection although not sterilizing immunity (Patterson et al., 2004). Here, in comparison to unimmunized macaques that naturally exhibited a degree of viremia control, we demonstrate the role of the vaccine regimen in induction of protective cellular immunity and maintenance of persistent immune responses, leading to durable protection manifested by low or undetectable viremia and preservation of CD4 T cells. Undoubtedly the low-level or unapparent infection of the protected macaques also contributed to maintenance of the immune response, but this process was initiated by a vaccine regimen that established viral control at a level that could be subsequently maintained by innate and adaptive host immune responses. This outcome provides support for the "threshold hypothesis" below which adequate control of SIV infection can be maintained (Ruprecht et al., 1996). The role of cellular immunity, including CTL activity and proliferative responses, in controlling HIV replication is well documented (Rosenberg et al., 1997, 1999). Studies in the SIV macaque system have similarly demonstrated the critical role of cellular immune responses in controlling viremia (Hel et al., 2002; Letvin et al., 1999).

While we have demonstrated that SIV-specific T cells contributed to durable protection against SIV_{mac251}, we have not elucidated specific protective mechanisms. Multiple mechanisms, including systemic and mucosal humoral and cellular immunity and innate immune responses, are likely involved. Given the outbred nature of study subjects, it may not be possible to uncover single "correlates of protection" in vaccine trials. Further, not all potential protective mechanisms were studied here. For example, soluble factors produced by CD8 T cells that suppress SIV infection (Cocchi et al., 1995; Mackewicz and Levy, 1992) may have played a role but were not investigated. Moreover, while we observed no correlation between antibody responses and protective outcome, all but two macaques, #7 and #9, exhibited functional SIV-specific antibodies that may have contributed to durable protection. The role of neutralizing antibody in mediating protection is well established (Mascola, 2003), and antibodies mediating ADCC have been correlated with reduced acute-phase viremia following SIV_{mac251} challenge (Gómez-Román et al., 2005). Both may have contributed to the protective outcomes seen here following re-challenge.

Overall, the strong durable protection against pathogenic SIV_{mac251} challenge observed in macaques immunized with multigenic replication-competent Ad-SIV recombinants and boosted with SIV envelope protein or an envelope protein subunit supports continued development of this vaccine approach. The potential advantage of the replication-competent Ad vector and other replicating vaccine vectors that also can target mucosal sites should be further investigated and exploited.

Materials and methods

Animals, re-challenge and sample collection

Eleven rhesus macaques (*Macaca mulata*) previously immunized with Ad5hr recombinants encoding SIV_{smH4}*env/rev*, SIV_{mac239}*gag* and/or SIV_{mac239}*nef* and boosted with native SIV_{mac251} gp120 or an SIV "peptomer" (Robey et al., 1995, 1996), a polypeptide representing the CD4 binding site of SIV_{mac251} gp120, were studied (Table 1). As described earlier in detail, all eleven macaques were previously strongly protected against a rectal SIV_{mac251} challenge (Patterson et al., 2004). A twelfth macaque, #38, that had undetectable viremia following the first SIV_{mac251} challenge was not available for re-challenge due to death, unrelated to SIV challenge, following a surgical procedure to collect biopsy samples. Two macaques (#34 and #39) that served as controls for the initial SIV_{mac251} challenge and had detectable viremia and low CD4+ T cell counts were included to compare the protective effect of prior immunization of the vaccinees with unimmunized macaques that gradually controlled viremia. Two naïve control macaques (#160 and #173) were included to monitor the infectivity of the administered SIV_{mac251} challenge. Animal housing and care were provided by Bioqual, Inc. in accordance with their Animal Care and Use Committee guidelines. All macaques were re-challenged intrarectally 53 weeks after the first SIV_{mac251} challenge with 10 animal infectious doses of pathogenic SIV_{mac251}. The SIV_{mac251} challenge stock, developed by Dr. Ronald Desrosiers, New England National Primate Research Center, and made available by Dr. Nancy Miller, Division of

AIDS, NIAID, NIH, was the same used for the initial virus challenge.

Peripheral blood mononuclear cells (PBMC) were purified from blood samples obtained 30 days before and throughout the re-challenge and CD8 depletion experiments using lymphocyte separation medium (ICN Pharmaceuticals, Inc.). Fresh cells were used for immunological assays. Plasma and sera were collected and stored at −70 °C. Inguinal LN biopsies and rectal pinch biopsies (10 pinches per animal) were obtained from immunized and control macaques 30 days before and on days 13 and 64 following CD8 depletion. The LNs were minced, and the released lymphocytes were washed 2 times in PBS containing 2% FCS and left overnight at 37 °C in standard R10 medium before use in intracellular cytokine staining (ICS) assays for IFN-γ. The rectal biopsies were minced in a Petri dish, then 15 ml of prewarmed R10 containing Collagenase II (50 U/ml, Sigma cat. #C-6885) was added and the tissues were placed in a belly dancer water bath for 15 min at 37 °C. The cells were washed by adding 15 ml of R10 or PBS and spinning at 1200 rpm for 10 min. The supernatant was removed, and the pellet was resuspended in 15 ml R10 plus Collagenase II (50 U/ml), and incubated 15 min, at 37 °C. The cells were washed, and the procedure was repeated 3 times. After the final washing, the cells were left overnight at 37 °C in R10. The next day, the cells were pushed through a 70-μm filter and counted prior to use.

Anti-CD8 antibody administration

Nine months after the SIV_{mac251} re-challenge, the 8 durably protected rhesus macaques and one SIV chronically infected, unimmunized macaque (#39) were administered the mouse-human chimeric monoclonal antibody to CD8 (cM-T807) (Schmitz et al., 1999a, 1999b). Another SIV chronically infected, unimmunized macaque (#34) was administered a control isotype antibody, Synagis (MedImmune, Inc., Gaithersburg, MD), in order to demonstrate that any change in viremia or CD8+ T cell counts was due to CD8 depletion rather than a response to the IgG itself. Antibodies were given subcutaneously at day 0 at 10 mg/kg of body weight, and at days 3 and 7 intravenously at 5 mg/kg of body weight.

Viral RNA measurement

Viral RNA in plasma was quantitated by using the nucleic acid sequence-based amplification technique (NASBA) as described previously (Romano et al., 2000). In this ECL-based detection assay RNA was quantitated with a standard sensitivity threshold of <2000 copies/input volume plasma. An assay with intermediate sensitivity (<500 copies/input volume of plasma, generally 1 ml) was also used where necessary. A real-time NASBA assay with a sensitivity of <50 copies/input volume was used to evaluate plasma from macaques that were consistently negative by the ECL-based assay. For the real-time assay, macaque plasma was clarified by centrifugation at 2300×*g* for three minutes. The clarified plasma (0.5 ml to 1 ml) was then centrifuged at 49100×*g* for 60 min, and the virus pellet was then lysed in 1 ml lysis buffer. Nucleic acid was isolated as described before (Romano et al., 2000), and the extracted RNA was used in real-time NASBA. The final NASBA reaction (20 μl) contained 5 μl of nucleic acid extract and 40 mM Tris, pH 8.5, 12 mM $MgCl_2$, 90 mM KCl, 5 mM dithiothreitol, 1 mM each dATP, dCTP, dGTP, dTTP; 2.0 mM each ATP, CTP, UTP; 1.5 mM GTP, 0.5 mM ITP, 0.1 μg/μl BSA, 1.5 M Sorbitol, 0.08 U RNase H, 32 U T7 RNA polymerase, 6.4 U avian myeloblastosis virus reverse transcriptase (AMV-RT), 0.01 μM SIV wild type (WT) molecular beacon probe, 0.1 μM SIV calibrator (Q) molecular beacon probe, 0.2 μM each of the two amplification oligonucleotides, and 15% DMSO. The SIV WT molecular beacon had the fluorophore FAM linked to the 5′end and a quencher linked to the 3′end, whereas the SIV Q molecular beacon had the fluorophore 6-ROX linked to the 5′end and a quencher linked to the 3′ end. For the assay all the components, except enzymes and BSA, were mixed and preincubated at 65 °C for 2 min. The reaction mixture was then cooled for 2 min at 41 °C and the BSA and enzymes added. The reaction mixture was then placed in the NucliSens EasyQ Analyzer (BioMerieux) and subjected to isothermal amplification at 41 °C for 90 min. An algorithm was then applied to calculate the SIV RNA copy number in the plasma sample. The assay had a lower limit sensitivity of 50 copies of RNA, and the SIV RNA load was expressed as viral RNA copies per 0.5 or 1 ml plasma.

For viral load quantification in tissues, total RNA was extracted from homogenized tissues by a method described previously (Chomczynski and Sacchi, 1987). Briefly, a small portion of the tissue was excised and lysed by homogenizing in a tube homogenizer with sodium citrate lysis buffer (pH 7.0) containing guanidine thiocyanate, sarcosyl, and β-mercaptoethanol. The lysed sample was treated with sodium acetate, extracted with phenol:chloroform (5:1) and RNA was precipitated with isopropanol. Extracted RNA was further purified by dissolving the pellet in sodium citrate lysis buffer and by reprecipitating with isopropanol. After washing with 70% ethanol, the RNA pellet was air dried, dissolved in water, and RNA content was quantitated by measuring 260-nm optical density. Quantitation of viral RNA was performed on a small aliquot (0.5 to 1 μg) of total RNA extract by NASBA. The assay had a lower limit sensitivity of 500 copies of RNA, and the SIV RNA load was expressed as viral RNA copies per microgram of total tissue RNA.

ELISPOT

The number of freshly isolated PBMC secreting IFN-γ in response to SIV Gag, Env, Nef, and Rev peptide pools was measured by a standard ELISPOT assay as described in detail elsewhere (Patterson et al., 2003). Env, Nef, and Rev peptides were 15-mers overlapping by 11 amino acids. The Gag peptides were 20-mers overlapping by 10 amino acids. Background counts in wells containing medium only have been subtracted from all reported values. A positive ELISPOT response was defined as equal to or greater than the number of background spot forming cells (SFC) plus 2 standard deviations. SFC resulting from

stimulation with 3 Env and 2 Gag peptide pools spanning the entire proteins were summed and reported as single values.

Proliferation assay

T cell proliferative responses of viably frozen PBMC to native endotoxin-free SIV gp120, SIV p27 (Advanced BioScience Laboratories, Inc.), and Aldrithiol-2-inactivated SIV_{mac239} (Ald-SIV; AIDS Vaccine Program, SAIC, NCI-Frederick, Frederick, MD) were measured by incorporation of ^{3}H thymidine (1 μCi/well) as described previously (Malkevitch et al., 2003).

Intracellular IFN-γ staining

After overnight incubation, fresh cells (1×10^{6}) from LN or rectal pinches were incubated at 37 °C for 1 h with a mixture of Env, Gag, or Nef peptide pools (1 μg/ml per peptide) or with no peptides as a negative control and then were treated with 10 μg/ml of Brefeldin A (Sigma) for the next 4–5 h at 37 °C in order to inhibit export of protein from the endoplasmic reticulum. The cells were washed two times with FACS buffer (PBS and 2% FCS) and stained for 20 min in the dark with 20 μl anti-CD3-FITC (BD Pharmingen), 10 μl anti-CD8-RPE (DAKO) and 20 μl anti-CD4-PerCP (BD Pharmingen), then washed twice with FACS buffer and fixed with 100 μl buffer A (Caltag) for 15 min at room temperature and washed again. The cells were permeabilized with 100 μl buffer B (Caltag) and stained with 5 μl of anti-IFN-γ APC (BD Pharmingen) for 15 min in the dark, at room temperature. The cells were washed once with FACS buffer and re-fixed with 450 μl 2% PFA. Flowcytometry acquisition was performed within the next 48 h. For LN cells, a minimum of 10,000–100,000 events were acquired, and for rectal cells, 3000–10,000 events.

Antibody assays

Serum binding antibodies to SIV_{mac251} gp120, p27, and Nef were determined by enzyme-linked immunosorbent assay (Buge et al., 1997). Binding titers were defined as the reciprocal of the serum dilution at which the absorbance of the test serum was twice that of the negative control serum diluted 1:50. IgG and IgA SIV_{mac251} gp120-specific binding antibodies in saliva and rectal secretions were assessed as previously described (Malkevitch et al., 2004). Neutralizing antibodies against laboratory-adapted SIV_{mac251} were evaluated in macaque sera as described previously (Zhao et al., 2003b). Fifty percent endpoint titers are reported.

Serum antibodies mediating ADCC were assessed using a flow-cytometric method as described previously (Gómez-Román et al., 2006). Human PBMC were used as effector cells. Killing of two types of target cells consisting of H9 cells infected with SIV_{mac251} and CEM.NKr cells coated with SIV_{mac251} gp120 was evaluated. Antibody titers are expressed as the reciprocal of the serum dilution at which percent killing was equal to or greater than percent killing of negative control samples plus three standard deviations.

Statistical analysis

The statistical method used to analyze ELISPOT responses was the fitting of a Bernoulli/lognormal mixture model with left censoring for the responses to the four peptides at each week (Moulton and Halsey, 1995). The Bernoulli component was assumed to have a logistic regression form. A threshold of 10 was set for each raw count adjusted for its background level (media mean plus 2 standard deviations). Fixed effects for the individual peptides and the groups and random effects for the correlation within macaques were included in the models. Differences between groups of macaques were assessed using the likelihood ratio test, except when all the adjusted counts were zero for one group, in which case the exact permutation distribution was used. The proliferative responses were analyzed by repeated measures analysis of variance, adjusting for the correlations over time in the responses to each antigen, after a power transformation for variance stabilization. The *P* values have been corrected for the multiple tests of the four antigens compared simultaneously between groups.

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